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Pathogenesis of *Salmonella*-induced enteritis

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Abstract

Infections with *Salmonella* serotypes are a major cause of food-borne diseases worldwide. Animal models other than the mouse have been employed for the study of nontyphoidal *Salmonella* infections because the murine model is not suitable for the study of *Salmonella*-induced diarrhea. The microbe has developed mechanisms to exploit the host cell machinery to its own purpose. Bacterial proteins delivered directly into the host cell cytosol cause cytoskeletal changes and interfere with host cell signaling pathways, which ultimately enhance disease manifestation. Recently, marked advances have been made in our understanding of the molecular interactions between *Salmonella* serotypes and their hosts. Here, we discuss the molecular basis of the pathogenesis of *Salmonella*-induced enteritis.

Key words
- *Salmonella typhimurium*
- Enteritis
- Diarrhea
- Salmonellosis

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Introduction

Salmonella serotypes have a broad host range and clinical manifestations that result from the combination between serotype and host species involved. Recently, a large amount of information on the molecular level of interactions between bacteria and host cells has allowed us to propose molecular mechanisms determining the pathologic and clinical manifestations of nontyphoidal *Salmonella* infections. For simplification, *Salmonella enterica* subsp. *enterica* ser. Typhimurium will be referred to hereafter as either *S. typhimurium* or serotype Typhimurium.

Salmonella infection is one of the most common food-borne infections worldwide. In the United States an estimated 1.41 million cases and more than 500 human deaths occur annually (1). Approximately 95% of the human *Salmonella* infections are food-borne, corresponding to approximately 30% of deaths caused by food-borne infections in the United States (1). *Salmonella* infection is even more detrimental in the developing world.

Experimental models for *Salmonella* infection

The mouse, which has been used for most of the studies addressing the various aspects of host-pathogen interaction in *S. typhimurium* infection, develops a systemic disease when infected with *S. typhimurium*, but no diarrhea. Murine infection with *S. typhimu-*

rium results in a disease that is similar to human typhoid fever caused by infection with serotypes Typhi, Paratyphi A, B, and C, which are host-restricted serotypes that infect only man and few other primate species. Thus, murine infection with *S. typhimurium* has been employed as a model for human typhoid fever, but not *Salmonella*-induced diarrhea. In contrast, calves infected with *S. typhimurium* develop a diarrheic disease with clinical manifestations similar to those observed in human infections, which also result in diarrhea with a low mortality rate (reviewed in Refs. 2 and 3).

An important aspect of the pathology of *Salmonella*-induced enteritis is the same pattern of inflammatory reaction developed by calves after *S. typhimurium* infection, which is characterized by a marked infiltration of neutrophils, also observed in non-human primates in experimental infections, and in human infections. In sharp contrast, mice infected with *S. typhimurium* develop an inflammatory response with predominance of mononuclear leukocytes, which is not associated with diarrhea (reviewed in Ref. 3).

Interestingly, although several bacterial genes are required for disease progression and expression in both calves and mice, some genes that play a role in the murine typhoid model are not required for enteropathogenesis in cattle and vice versa (4). Many of the *Salmonella* virulence genes are clustered in certain areas of the chromosome known as "*Salmonella* pathogenicity islands" (SPI). To date, five SPI have been described and two of them, SPI-1 and SPI-2, encode type III secretion systems. The SPI-1-encoded type III secretion system translocates effector proteins into the cytosol of host cells. This system is required for invasion of nonphagocytic host cells (5) and enteropathogenesis (6), while the SPI-2-encoded type III secretion system is required for intracellular survival in murine macrophages (7). In the murine typhoid model, *S. typhimurium* strains having mutations in SPI-1 and SPI-2 are 50-fold and >10,000-fold attenuated, respectively, after oral infection (5,7). In contrast, SPI-2 does not play a major role in enteropathogenesis, whereas SPI-1 mutants are nonpathogenic for calves (6) as opposed to just a mild attenuation of these SPI-1 mutants in the mouse.

Invasion of epithelial cells by *S. typhimurium*

A remarkable aspect of *Salmonella* pathogenesis is its ability to invade nonphagocytic cells in a process that morphologically resembles phagocytosis. M cells located in the follicle-associated epithelium in the Peyer's patches are the primary intestinal epithelial cell type targeted for invasion by *Salmonella* in the mouse (8). In cattle, *S. typhimurium* is able to invade both M cells and enterocytes with no predilection for a particular cell type (9).

Upon contact with intestinal epithelial cells, *S. typhimurium* translocates bacterial effector proteins into the host cell cytosol via the SPI-1-encoded type III secretion system previously discussed. Some of these proteins have kinase, phosphatase, or actin-binding activity, and once in the epithelial cell cytosol, they alter host cell signaling pathways that promote changes in the cytoskeleton, with consequent bacterial internalization and changes in host gene expression (reviewed in Ref. 10). Mutant strains of *S. typhimurium* lacking structural components of the SPI-1-encoded type III secretion system, secreted proteins, or SPI-1 transcriptional regulators are unable to invade epithelial cells (11).

Salmonella senses environmental factors such as oxygen concentration, osmolarity, and pH that determine the expression of invasion genes in the intestinal lumen when their products are required for invasion of intestinal epithelial cells. The effects of these environmental factors are mediated by regulation of expression of the transcriptional

regulator HilA (12). HilA-dependent regulation of expression is not restricted to SPI-1, since SPI-4- and SPI-5-encoded genes were found to be regulated by HilA, whose expression is regulated by SirA (13).

Studies in the early 1990's determined the morphologic features and dynamics of the interaction between *S. typhimurium* and intestinal epithelial cell monolayers. Shortly after *Salmonella* enters in contact with the apical surface of the epithelial monolayer, the epithelia develop cytoplasmic projections with disruption of the underlying cytoskeleton, and intracellular bacteria are detected 30 min after infection. Two hours post-infection, free bacteria are detected on the basolateral side of the monolayer (14). Additional studies indicated that *S. typhimurium* grown under conditions that favor expression of invasin induce morphologic changes in epithelial cells as quickly as 40 s after contact (15), which are associated with recruitment of cytoskeletal components (16). These morphological and cytoskeletal changes, characterized by formation of ruffle-like structures, mediate bacterial internalization into epithelial cells (17). As shown in Figure 1, similar morphologic changes occur in calves infected with *S. typhimurium in vivo*. SipC is an SPI-1-encoded protein that acts as a translocase and is translocated itself into the host cytosol via the SPI-1-encoded type III secretion system. This protein bundles actin filaments and nucleates actin polymerization *in vitro*, which results in cytoskeletal rearrangements *in vivo* (18). SipA, which is not required for invasion, binds to F-actin inhibiting depolymerization (19). Thus, SipC is essential for actin nucleation and bundling of actin filaments whereas SipA acts by enhancing the efficiency of this process (20).

Although *sopE* is absent in many *S. typhimurium* strains, a homologue, *sopE2*, is present in all strains of *S. typhimurium* (21). Like SopE, SopE2 is also a guanine nucleotide exchange factor for Cdc42 and plays a role recruiting the actin-nucleating complex Arp2/3 to the membrane ruffles (21). Therefore, SopE2 is required for optimal invasion of cultured epithelial cells by *S. typhimurium* (21). SptP, a *Salmonella* protein that acts as a GTPase-activating factor for Rac-1 and Cdc42 and is also delivered into the epithelial cell cytosol via the SPI-1-encoded type III secretion system, has been shown to disrupt the actin cytoskeleton. Therefore it acts by reversing the cytoskeletal changes induced by the bacteria during invasion, restoring the normal cytoskeletal structure (22). The effect of SptP antagonizing the action of other bacterial effector proteins clearly indicates that *S. typhimurium* is able to finely regulate cellular pathways in favor of its own purposes.

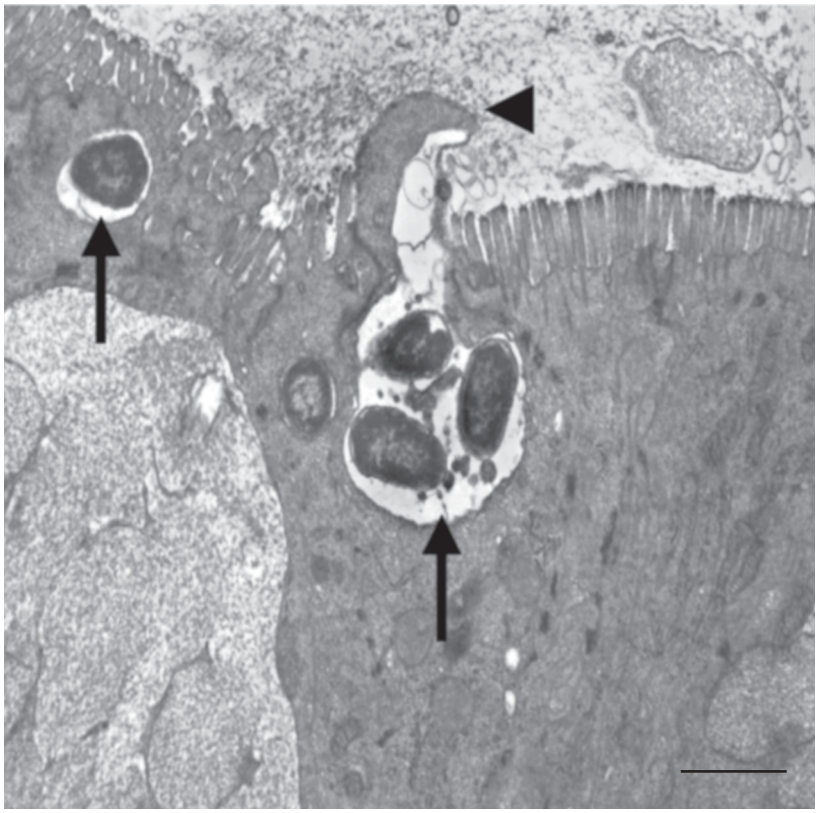


Figure 1. Transmission electron micrograph showing invasion of bovine enterocytes in the Peyer's patches by *Salmonella typhimurium*. Intracellular bacteria are located within membrane bound vacuoles (arrows). Bacteria being internalized by cytoplasmic projections of the apical surface of the enterocyte (arrowhead). Bar = 1 µm.

Inflammatory response during *S. typhimurium* infection

Although the complete mechanism of *Salmonella*-induced diarrhea is still not clear, some previous reports indicate that it is distinct from secretory diarrheas such as those caused by cholera toxin (23,24). As discussed above, infection of calves with *S. typhimurium* results in enteritis in which neutrophils are the primary inflammatory cells involved. On the other hand, the mouse, which does not develop diarrhea, responds to the infection mostly with a mononuclear infiltrate in the intestine (3). Furthermore, experimental depletion of the polymorphonuclear leukocyte pool by administration of nitrogen mustard to rabbits results in a significant decrease in intestinal fluid secretion induced by *S. typhimurium* infection (23). In addition, administration of indomethacin, an anti-inflammatory agent, completely abolishes fluid secretion in rabbit intestinal loops inoculated with *S. typhimurium* (24). Therefore, neutrophils are proposed to play a very important role in the pathogenesis of *Salmonella*-induced diarrhea. Some of our recent experimental findings corroborate this hypothesis since the inflammatory response, characterized by neutrophil infiltration, precedes intestinal fluid secretion after infection with *S. typhimurium* in calves (25). Several *Salmonella* virulence factors required for enteropathogenicity in calves, which are involved in eliciting neutrophil influx, are also required for fluid secretion (26,27), reinforcing the significance of neutrophils in this process.

Following oral infection, intestinal epithelial cells are the first barrier to be crossed by *S. typhimurium* in order to invade and colonize the intestinal tissues and other organs. Current data indicate that epithelial cells play an important role in the outcome of infection by influencing the host inflammatory response. Supporting this notion, a study demonstrated that *Salmonella* strains and serotypes that elicit enteritis and diarrhea are also able to induce transepithelial signaling for neutrophil migration across epithelial cell layers, whereas strains that do not cause diarrhea failed to trigger neutrophil transepithelial migration (28). Invasion of cultured epithelial cells by bacteria, including *Salmonella*, results in expression and secretion of interleukin 8 (IL-8), a chemoattractant for neutrophils (29). *Salmonella*-induced IL-8 secretion by epithelial cells is dependent on the mitogen-activated protein kinase pathway and activation of the transcription factor NF-κB and requires a functional SPI-1-encoded type III secretion system (30). Cultured epithelial cells also respond to *S. typhimurium* invasion with an increase in the cytosolic concentration of calcium, which is absolutely required for NF-κB activation and IL-8 expression (31).

Infiltration of neutrophils into the lamina propria occurs shortly after infection with *S. typhimurium*, which is followed by a massive migration of neutrophils through the epithelium into the intestinal lumen (9,25). Thus, one can expect that chemoattractants for neutrophils are secreted into the intestinal lumen at the early stages of infection. Interestingly, experiments with polarized intestinal epithelial cell monolayers indicate that IL-8 is secreted at the basolateral aspect of the epithelium, which implies that the role of IL-8 is primarily recruitment of neutrophils to the subepithelial space rather than transepithelial migration into the intestinal lumen (32). Further experiments have led to the identification of a pathogen-elicited epithelial chemoattractant (PEEC) bioactivity, which is released in a polarized fashion towards the apical aspect of the epithelial monolayer. Once secreted on the apical side of the epithelial cell, PEEC induces direct migration of neutrophils across cultured intestinal epithelial cell monolayers (33). PEEC has a 1- to 3-kDa mass, stimulates neutrophils via a pertussis toxin-sensitive receptor and elicits the Ca^{2+} signal, but its molecular nature is

still uncharacterized (33). Actual invasion of epithelial cells is not required for induction of epithelial promotion of neutrophil transepithelial migration since treatment of the monolayers with cytochalasin D, which blocks *S. typhimurium* invasion, does not reduce the promotion of neutrophil migration (34). Importantly, although IL-8 and PEEC act in concert to promote neutrophil migration through the lamina propria and epithelia, respectively, secretion of these two chemotactic factors is mediated by distinct signaling pathways. In contrast to IL-8 expression and secretion, PEEC activity is not dependent on NF-κB activation (34). It has been recently demonstrated that the bacterial protein SipA, an SPI-1-encoded effector protein translocated into the cytosol of the host cell via the SPI-1 type III secretion system, is sufficient to trigger neutrophil transepithelial migration in cultured intestinal epithelial monolayers (35). Another *Salmonella* protein secreted by the SPI-1-encoded type III secretion system, SopA, is also involved in the induction of neutrophil transepithelial migration (36).

Although some recent studies have addressed *in vivo* cytokine production in response to *Salmonella* infection, most of these studies were performed on mice. We have demonstrated that there is a marked increase in expression of CXC chemokines such as IL-8, GROα/γ and GCP2, and the proinflammatory cytokine IL-1ß in bovine Peyer's patches as early as 1 h post-infection, increasing continuously until at least 5 h post-infection (9). Interestingly, anti-inflammatory cytokines such as IL-4 and the IL-1 receptor antagonist (IL-1Ra) are also upregulated in bovine Peyer's patches *in vivo* after infection with *S. typhimurium* (9).

Salmonella-induced host cell death

Although apoptosis has been defined classically as a form of cell death that does not elicit an inflammatory reaction, under specific conditions this process may ultimately act as a proinflammatory signal. Several groups have reported that murine macrophages and macrophage-like cell lines undergo cell death when infected with *S. typhimurium* (37-39). A previous report indicated that *Salmonella*-induced macrophage apoptosis is associated with marked IL-1 release (40). Thus, since IL-1 is a potent pro-inflammatory cytokine, this was the first indication of a possible link between *Salmonella*-induced cell death and inflammation. *Salmonella*-induced macrophage cell death is largely due to expression of genes associated with invasion, since mutant strains lacking functional SPI-1 or grown under conditions that prevent SPI-1 expression do not cause rapid cell death after infection of macrophages (37,39,41,42), although SPI-1-independent cell death has also been described (38,42,43). Further investigation has led to the identification of the SipB protein as the bacterial effector responsible for induction of apoptosis (44). SipB is translocated into the host cell cytosol via the SPI-1-encoded type III secretion system, where it binds to and activates caspase-1, an intracellular cysteine protease also known as IL-1ß converting enzyme. Once activated, caspase-1 cleaves and activates IL-1ß (44). Caspase-1 is also responsible for triggering apoptosis in *Salmonella*-infected macrophages, since a specific caspase-1 inhibitor blocks this mechanism of cell death (44). Infection of macrophages with *S. typhimurium* also results in degradation of the host protein Raf-1 in a SipB- and caspase-1-dependent manner, which favors the cytotoxic effect of SipB since Raf-1 acts by antagonizing the caspase-1-mediated cell death (45). Thus, *Salmonella*-induced macrophage apoptosis results in release of active IL-1ß, which is thought to play a significant role in *Salmonella*-elicited inflammation. This mechanism appears to be conserved since a similar mechanism of cell death, mediated by SipB and caspase-1 activity, occurs in bovine mac-

rophages infected with *S. typhimurium* (42).

Although all the initial papers described *Salmonella*-induced cell death as apoptotic in nature, more recent publications argue that it is a necrotic rather that an apoptotic mechanism of cell death. The conclusions of these reports are based on either failure to detect DNA fragmentation and morphologic features of apoptosis (46) or on the effect of glycine blocking *Salmonella*-induced cytotoxicity (47). However, while the definition of a proper classification and terminology for this mechanism is still debatable, the requirement for caspase-1 activation and the proinflammatory nature of this mechanism is a consensus among different laboratories and has lead to the proposition of "pyroptosis" as a new term to describe proinflammatory programmed cell death (48).

In spite of the increasing amount of information on the interaction between *Salmonella* and macrophage in cell culture systems, there are few data available regarding the significance of macrophage cell death and its putative proinflammatory effect on the outcome of *Salmonella* infections *in vivo*. The ability of *S. typhimurium* to induce macrophage cell death has been demonstrated *in vivo* in mice intravenously inoculated with small infectious doses (49). In addition, caspase-1 is required for colonization of Peyer's patches and induction of systemic infection in mice orally inoculated with *S. typhimurium* (50). Although quite valuable, these data are not applicable to the pathogenesis of *Salmonella*-induced diarrhea. Indeed, we have demonstrated that *Salmonella*-induced host cell death is not sufficient to trigger the inflammatory response after *S. typhimurium* infection in calves (25).

Salmonella virulence factors involved in enteropathogenesis

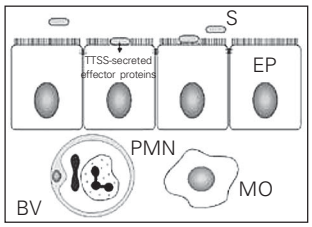
There is clearly a difference between *Salmonella* genes required for virulence in mice, where the *Salmonella* virulence plasmid and SPI-2-encoded genes are essential for virulence, and those involved in eliciting diarrhea, in which SPI-1-encoded genes are essential but SPI-2 and the *Salmonella* virulence plasmid play only minor roles (6,13,26, 51,52). Disruption of the SPI-1 type III secretion decreases or abolishes the ability of *S. typhimurium* to invade the intestinal epithelium, which correlates with its ability to elicit an inflammatory response and thereby induce diarrhea (6,13,26,51).

A fifth SPI has been identified and linked to the pathogenesis of diarrhea (27,53). An SPI-5-encoded gene, *sopB*, has been extensively studied. SopB, also known as SigD, is secreted via the SPI-1-encoded type III secretion system and its expression is dependent on the regulator *sirA*, which is also an activator of the SPI-1 regulator *hilA* (54). A *sopB* mutant of *S. dublin* has a significantly reduced ability to elicit inflammation and fluid secretion in bovine ligated ileal loops in spite of displaying wild-type levels of invasion in the Peyer's patches (27). Similar results were observed with a *sopB* mutant of *S. typhimurium* (25). SopB is an inositol phosphate phosphatase that hydrolyzes phosphatidylinositol 3,4,5-triphosphate, which is an inhibitor of chloride secretion. In addition, SopB hydrolyzes inositol 1,3,4,5,6 pentakisphosphate, generating inositol 1,4,5,6 tetrakisphosphate (55), which may be involved in increasing chloride secretion (56). Thus, SopB is thought to mediate fluid secretion by increasing chloride secretion. However, changes in chloride secretion alone are not compatible with the pathologic features of *Salmonella*-induced diarrhea, which is associated with a severe acute neutrophilic infiltration. The most significant events in the pathogenesis of *Salmonella*-induced enteritis are illustrated in Figure 2. Interestingly, SopB also affects host cell signaling pathways that may be involved in regulation of cytokine expression such as activation of the serine-threonine kinase Akt (57). The *Salmonella* protein SopD, which is also se-

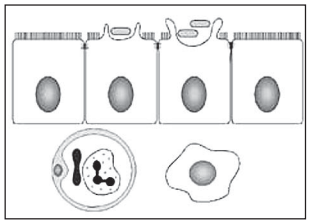
creted in an SPI-1-dependent manner, has an additive effect to SopB in the induction of enteritis (58), whereas SopA influences the inflammatory response by a mechanism distinct from SopB and SopD. SopA is involved in induction of transepithelial migration of neutrophils, a phenomenon that is not influenced by SopB or SopD (36). Recent experimental findings from our laboratory indicate that the secreted effectors SipA, SopA, SopB, SopD, and SopE2 act in concert to induce diarrhea, since a strain lacking all of these genes (Δ*sipAsopABDE*) had additive attenuation when compared to the single gene mutants. The quintuplet mutant (Δ*sipAsopABDE*) was as attenuated as a mutant with a defective SPI-1-encoded type III secretion system in the bovine ileal loop model (59). The role of *Salmonella* virulence genes in enteropathogenesis is summarized in Table 1.

Salmonella-induced diarrhea is an effi-

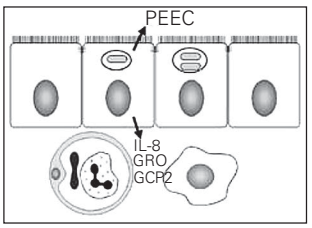
Figure 2. Schematic representation of the pathogenesis of *Salmonella*-induced enteritis, with the most significant events described from A through H.



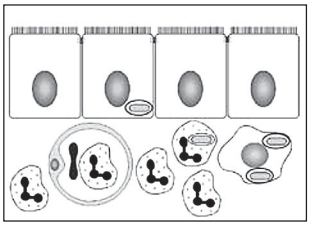
A. *Salmonella* (S) attaches to the apical surface of the intestinal epithelia (EP) and injects the effector proteins through the SPI-1-encoded type III secretion system (TTSS) into the host cell cytosol. Resident macrophages (MO), a blood vessel (BV), and an intravascular neutrophil (PMN) are located in the lamina propria.



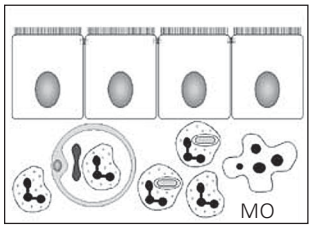
B. *Salmonella* effector proteins, particularly SipA and SipC, modulate actin polymerization resulting in ruffling formation at the apical surface of the epithelial layer. This event takes place in both M cells and enterocytes as early as 15 min post-inoculation.



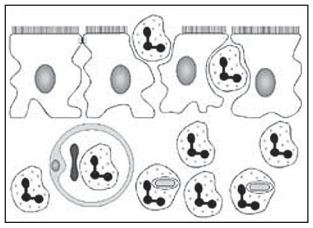
C. In addition to cytoskeletal remodeling, some of the *Salmonella* effector proteins trigger nuclear responses that ultimately result in increased expression of chemotactic factors. Interestingly, pathogen-elicited epithelial chemoattractant (PEEC) is secreted toward the apical side whereas IL-8 is secreted towards the basolateral side of the epithelial layer.



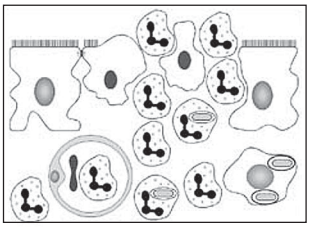
D. In response to chemotactic stimuli, there is infiltration of neutrophils in the lamina propria. At 1 h post-infection *Salmonella* reaches the basal portion of the epithelial layer and undergoes phagocytosis by neutrophils and macrophages.



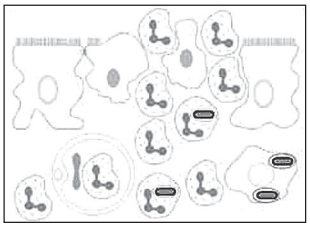
E. At least in cell culture systems, *Salmonella* induces macrophage (MO) cell death, which is triggered by SipB binding and activation of caspase-1. IL-1b is then released and may enhance the inflammatory reaction. In sharp contrast, neutrophils do not undergo cell death due to infection with *Salmonella*.



F. As the inflammatory reaction progresses, neutrophils migrate through the epithelial layer, with accumulation of inflammatory cells and protein-rich fluid into the intestinal lumen. These events develop from 1 to 3 h post-inoculation.



G. The overwhelming inflammatory reaction results in massive transepithelial migration of neutrophils, which causes epithelial detachment from the basal membrane, favoring fluid secretion into the intestinal lumen and diarrhea.



H. Due to the release of proteases and other mediators from inflammatory cells, extensive necrosis of the superficial mucosa takes place at 24 to 48 h post-infection. The resulting necrotic debris provide an adequate substrate for bacterial growth facilitating shedding and environmental contamination.

Table 1. Role of virulence genes in enteropathogenesis.

Effector gene	Required for fluid accumulation in bovine ligated ileal loops	Virulence during oral infection of calves at 10^{10} CFU/animal (dead/total number infected)	Reference
sipB (*sspB*)	yes	0/4	59
sipC (*sspC*)	nd	0/2	60
sipD (*sspD*)	nd	0/2	60
sipA (*sspA*)	yes	3/4	59
sopA	yes	4/4	59
sopB	yes	4/4	6,25
sopD	yes	3/4	59
sopE2	yes	4/4	59
sspH1	no	nd	59
avrA	no	nd	59
sptP	no	3/4	60
sipAsopABDE2	yes	1/4	59

nd = not determined, CFU = colony-forming units.

cient way of spreading the organism in the environment. Recently, a gene, named *shdA*, found only in strains of *Salmonella* adapted to warm blooded-organisms (subspecies I) has been demonstrated to be involved in prolonged shedding of *S. typhimurium* in mice. Mutation of *shdA* causes a decrease in the number of organisms shed in feces and the duration of shedding (61). Subsequent studies indicated that the *shdA* gene product binds to extracellular matrix proteins, particularly fibronectin (62).

From these discussions of the mechanisms of *Salmonella*-induced diarrhea, it is exceedingly clear that there is an intensive and intricate series of highly regulated adaptive gene expression events by both the host and the *Salmonella* microbe. Unraveling the intricacies of the molecular basis and the regulation of these interactions holds great promise for developing new vaccination strategies as well as improved therapeutic rationales.

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